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Functional and biochemical association of Hck with the LIF/IL-6 receptor signal transducing subunit gp130 in embryonic stem cells

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The role played by the Src-related tyrosine kinase, Hck, in embryonic stem (ES) cell differentiation was investigated by replacing a conserved C-terminally located tyrosine with phenylalanine by gene targeting. Targeted ES cells display a 7- to 9-fold elevation in constitutive Hck kinase activity and require ~15 times less leukaemia inhibitory factor (LIF) than parental ES cells to maintain their stem cell character *in vitro*. We also demonstrate a rapid and transient increase in Hck tyrosine kinase activity in parental ES cells stimulated by LIF and, finally, show that Hck is physically associated with gp130, an affinity converter and signal transducing component of the LIF receptor. Thus, these results provide biological and biochemical evidence that Hck participates in signal transduction from the LIF receptor.

Key words: gene targeting/gp130/Hck/LIF receptor/signal transduction

Introduction

The coordinated control of cellular growth and differentiation in eukaryotes is achieved, in part, through the activation of intracellular biochemical networks in response to extracellular signals. The process is initiated when extracellular signalling molecules bind to their cognate cell surface receptors resulting in a cascade of biochemical events that culminates in changes in gene expression, and the promotion or inhibition of cellular growth and/or differentiation. Amongst the most important of the various classes of intracellular signalling molecules are the protein tyrosine kinases. In some cases the cell surface receptors themselves encode intrinsic tyrosine kinase activity that is activated upon ligand binding. Other cell surface receptors, including those for many cytokines, lack intrinsic tyrosine kinase activity while ligand-dependent tyrosine phosphorylation of specific substrates is often still observed [for reviews see Miyajima (1992) and Lord *et al.* (1991)]. Recently, some members of this class of receptors have been shown to exist in the same physical complexes with one or several members of the Src family of protein tyrosine kinases which are activated upon binding of the ligand to the receptor (Veillette *et al.*, 1989; Samelson *et al.*, 1990; Burkhardt *et al.*, 1991; Hatakeyama *et al.*, 1991; Kobayashi *et al.*, 1993).

In the present study we have attempted to establish a role for one member of the Src family of protein tyrosine kinases, Hck, in signal transduction (Holtzman *et al.*, 1987; Quintrell *et al.*, 1987; Ziegler *et al.*, 1987). While expression of Hck is primarily restricted to the haemopoietic system, and in particular to cells of the myeloid and B-lymphoid lineages (Holtzman *et al.*, 1987; Quintrell *et al.*, 1987; Ziegler *et al.*, 1987), we have recently shown that Hck is also expressed in undifferentiated ES cells and that its expression is reduced substantially when the cells are allowed to differentiate *in vitro* following withdrawal of leukaemia inhibitory factor (LIF) (M.Ernst, Steve Ralph and A.R.Dunn, unpublished observations). Like other members of the Src family of protein tyrosine kinases, Hck is negatively regulated by phosphorylation of a conserved C-terminally located tyrosine residue [Y499 in murine Hck (Holtzman *et al.*, 1987)]. A 10- to 20-fold increase in tyrosine kinase activity was observed in cells transfected with cDNAs of Src-related tyrosine kinases in which the C-terminal tyrosine had been replaced by phenylalanine (Y499F) (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Amrein and Sefton, 1988; Kawakami *et al.*, 1988; Marth *et al.*, 1988; Ziegler *et al.*, 1989). In order to explore possible functions for Hck in ES cells (and ultimately in transgenic mice), we have employed the 'hit-and-run' gene targeting strategy (Hasty *et al.*, 1991) to alter the specific activity of the protein tyrosine kinase encoded by the *hck* gene in ES cells by replacing HckY499 with phenylalanine (HckY499F). In this strategy, all of the *cis*-acting elements of the *hck* gene that collectively serve to regulate the temporal and spatial patterns of its expression remain intact and thus any alteration in cellular physiology can be confidently attributed to disregulated Hck tyrosine kinase rather than to its ectopic or unscheduled expression.

As expected, the targeted undifferentiated ES cell lines display elevated Hck tyrosine kinase activity. Intriguingly, the concentration of LIF required to maintain the stem cell character of targeted ES cell lines is ~15 times lower than that for parental cells when cultured under otherwise identical conditions. Thus, activated Hck transmits a LIF receptor (LIFR) signal that retards differentiation raising the possibility that signalling through the LIFR is, in part, mediated through Hck. This notion is reinforced by our observation that Hck tyrosine kinase activity is rapidly and transiently increased following stimulation of ES cells with LIF. We further show by co-immunoprecipitation, that Hck exists in the same physical complex as the LIFR complex and is associated with gp130, the common affinity converter and signal transducing component of the functional LIFR (Gearing *et al.*, 1992; Ip *et al.*, 1992) and interleukin 6 receptor (IL-6R) complexes (Hibi *et al.*, 1990).

Results

'Hit-and-run' gene targeting strategy

We employed the 'hit-and-run' gene targeting strategy in order to introduce a subtle mutation into the *hck* gene without retaining any prokaryotic sequences in the mammalian genome (Hasty *et al.*, 1991). An 'insertion-type' targeting vector, p*HCK*499F, was designed to contain minimal amounts of sequence homology to exons encoding the 3'-end of the *hck* gene (Figure 1) (Holtzman *et al.*, 1987; Ziegler *et al.*, 1991) in order to minimize the chance of acquiring undesired, secondary mutations in the coding sequences of the *hck* gene during homologous recombination. A PCR-based strategy was used to introduce a single A to T nucleotide substitution in codon 499 (tyrosine to phenylalanine) as well as additional conservative nucleotide substitutions, in the immediately adjacent sequence, to generate a novel oligonucleotide binding site for a diagnostic PCR primer (primer 2, Figure 1). The targeting vector also contained a hypoxanthine phosphoribosyl transferase (HPRT) minigene under the control of the phosphoglycerokinase (PGK) promoter cloned immediately 5' of the *hck* genomic sequence contained within p*HCK*499F. The vector was linearized inside the region of homology and electroporated into E14TG2a ES cells, an HPRT-deficient cell line (Hooper *et al.*, 1987), which were subsequently cultured in growth medium containing hypoxanthine, thymidine and aminopterine (HAT). Where homologous integration occurs, a duplication of the homologous sequence is generated which, together with the HPRT minigene, can subsequently be excised by spontaneous intrachromosomal recombination. Therefore, the loss of the HPRT expression cassette is only likely to occur in cells that hitherto contained the duplication and thus medium containing 6-thioguanine (6-TG) also selects against cells containing additional randomly integrated copies of the targeting vector. The strong selection by 6-TG negates the need to screen the intermediate population of HAT^r cells for homologous integration. Since we have not isolated targeted HAT^r cell lines, the targeting frequency was estimated to be 5×10^{-3} based on the frequency of 6-TG^r clones recovered.

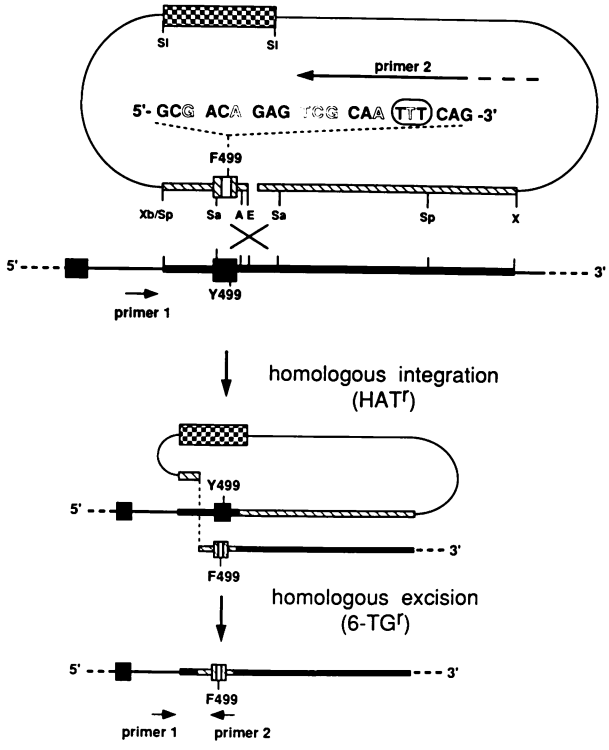

Fig. 1. The p*HCK*499F targeting vector used in the 'hit-and-run' gene targeting strategy for the introduction of the Y499F mutation in the *hck* gene. The mutation consists of a single A to T substitution in codon 499 (circled) and six additional silent nucleotide substitutions (open letters). The targeting vector contains 0.8 kb of homologous sequence 5' of the Y499F mutation and 6.6 kb of homologous sequence at its 3' side (hatched box) as well as the PGK-HPRT minigene (chequered box) in the vector backbone. Following stable integration ('hit') of the p*HCK*499F vector, a subset of the HAT^r cells contains a duplication of the genomic sequences as the result of homologous integration. Subsequently, intrachromosomal recombination can occur spontaneously within the duplicated sequence ('run') thereby excising the interstitial vector sequence, including the HPRT minigene, and the duplicated *hck* sequences. Although all reverted cells become 6-TG^r, the Y499F substitution is retained in the targeted allele only if the recombination within the intermediate Holliday structure occurs distal to the interstitial vector sequence. Primer 1, which maps to an *hck* sequence 5' of the boundary of the targeting construct, and primer 2 are used in a PCR-based strategy to screen 6-TG^r cells for the presence of the mutation. Squares represent exons 12 and 13 of the murine *hck* gene. Black squares and thick lines refer to genomic sequences at the *hck* locus. Stippled squares and hatched lines indicate homologous sequences in the targeting vector p*HCK*499F while thin lines represent bacterial plasmid sequences. Restriction sites: A, *Apa*I; E, *Eco*RI; Sa, *Sac*I; Sl, *Sal*I; Sp, *Spe*I; X, *Xho*I; Xb, *Xba*I.

A subset of 6-TG^r colonies contains the hckY499F mutation

Depending on the site where crossing-over takes place during resolution of the recombination structure (Figure 1), only a subset of 6-TG^r cells would be expected to contain the Y499F substitution. Therefore, we screened 6-TG^r ES colonies for the presence of the mutation by PCR using oligonucleotides corresponding to the mutated locus (primer 2) and to a sequence 5' of the boundary of the targeting vector (primer 1). Of the 6-TG^r colonies screened in this way, about one in five displayed the diagnostic 800 bp fragment (data not shown). The presence of the Y499F mutation in ES cells was confirmed by nucleotide sequence analysis of DNA amplified from three independently derived targeted ES cell lines (K5.19, K6.2 and K7.9) using PCR primers 1 and 3 that span exon 13 (Figure 2). With the exception of the predetermined mutations around amino acid residue 499, and a single polymorphism between BALB/c (targeting vector) and 129/Ola (ES cells) DNA at position 488 (CTG→CTT), the nucleotide sequence of the targeted exon was identical to the wild-type counterpart, showing that no secondary mutation within the coding region had been acquired during homologous recombination.

Targeted ES cell lines have elevated levels of Hck tyrosine kinase

To determine whether the Y499F mutation resulted in altered Hck tyrosine kinase activity, we compared Hck autophosphorylation in the three independently derived targeted cell lines (K5.19, K6.2 and K7.9) with that of three subclones of parental E14TG2a cells (E14TG2a.1, E14TG2a.4 and E14TG2a.5). To ensure that equal amounts of Hck protein were used in the *in vitro* protein kinase assay, cultures of targeted and parental ES cell lines were first metabolically labelled with [^{35}S]methionine/[^{35}S]cysteine

and Hck protein was immunoprecipitated with an Hck-specific antiserum (Figure 3A). Equal amounts of Hck-associated ^{35}S radioactivity were then used for the *in vitro* phosphorylation assay in the presence of [γ-^{32}P]ATP. The two species of Hck (p56^{hck} and p59^{hck}) represent discrete translation products arising from selective utilization of alternative translational initiation codons within the *hck* mRNA (Lock *et al.*, 1991). Targeted cell lines displayed an ~8-fold higher level of Hck autophosphorylation than the parental E14TG2a cell lines (Figure 3B). Similarly, Hck recovered from targeted cells exhibited a 4- to 6-fold increase in its ability to phosphorylate the exogenous substrate enolase (Figure 3C).

ES cells with targeted HckY499F mutation have decreased requirements for LIF

Our observation that the level of Hck expression falls dramatically during differentiation of ES cells following withdrawal of LIF from the culture medium, raised the possibility that Hck participates in signal transduction from the LIFR. In the first instance, we sought to determine whether the targeted ES cell lines showed an altered requirement for LIF in order to remain undifferentiated. Figure 4 shows that the targeted HckY499F ES cell lines require ~15 times less LIF than parental E14TG2a cells in order to maintain a comparable proportion of morphologically undifferentiated, densely packed ES cell colonies.

To characterize the altered response of targeted cells to LIF in more detail, ES cells were subjected to a 'LIF-rescue' assay to determine the proportion of ES cells that remain undifferentiated following an intermediate 'LIF-starvation' period in sub-optimal concentrations of LIF (2 U/ml) versus cultures maintained in optimal LIF concentrations (60 U/ml). As shown in Figure 5, the proportion of ES cells that remained morphologically undifferentiated following 'LIF-starvation' for >20 h, was significantly greater in each of the three targeted cell lines than in the three subclones of parental ES cells. After 60 h of 'LIF-starvation' of parental cells with 2 U/ml of LIF for example, colonies of morphologically undifferentiated cells could only be 'rescued' at a frequency of ~30% in parental cells as

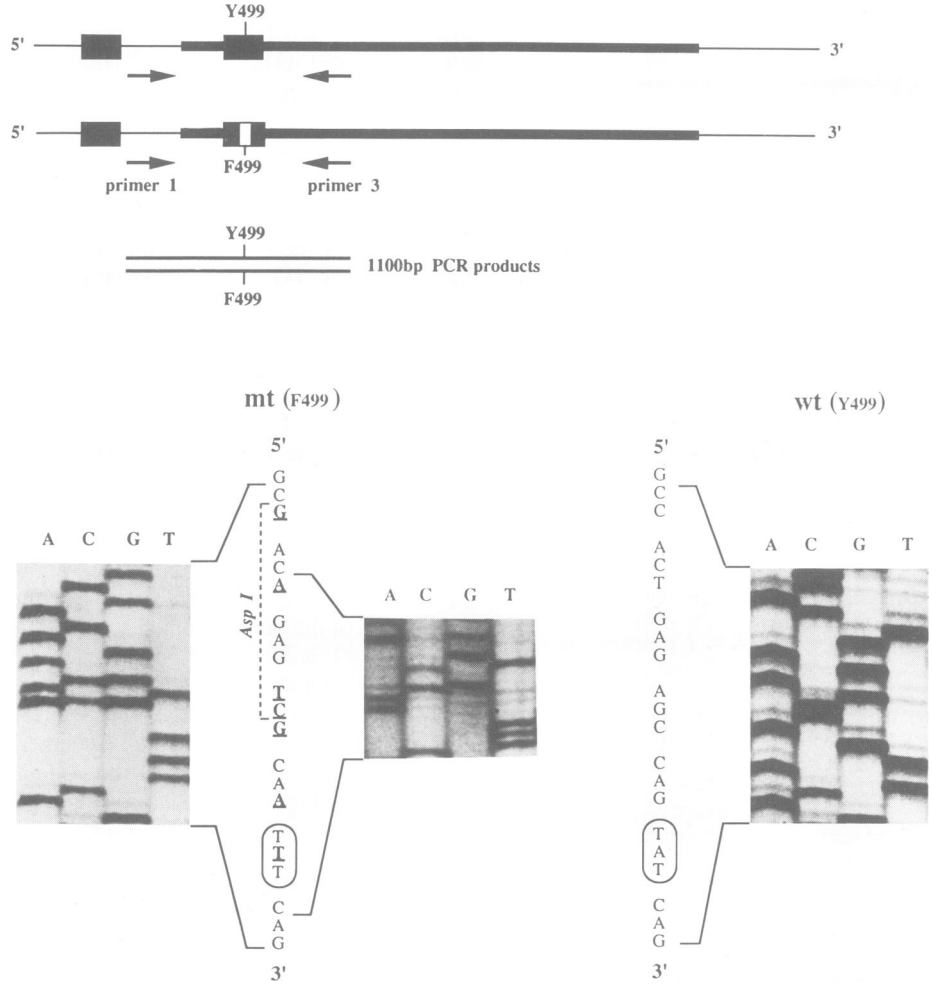

Fig. 2. Confirmation of the nucleotide sequence of the targeted Y499F mutation in ES cells. PCR primers 1 and 3 were used in conjunction with DNA prepared from the PCR-positive ES cell line K6.2 to amplify exon 13 of the *hck* gene. Since these primers will prime from both alleles and thus produce a mixture of products corresponding to the mutated and the wild-type allele, the subcloned PCR products were analysed for the presence of the newly introduced *Asp*I site. Comparison of the sense strand nucleotide sequence obtained from the wild-type allele (**right**) with the sense (**left**) and antisense (**middle**) strand sequence from the targeted allele confirms the authenticity of the HckY499F mutation in the targeted allele. The mutated nucleotides are underlined, and the Y499F mutation is encircled. Boxes represent coding *hck* exons, thick lines correspond to untranslated sequences which were part of the targeting construct, and thin lines mark untranslated sequences outside of the targeting vector.

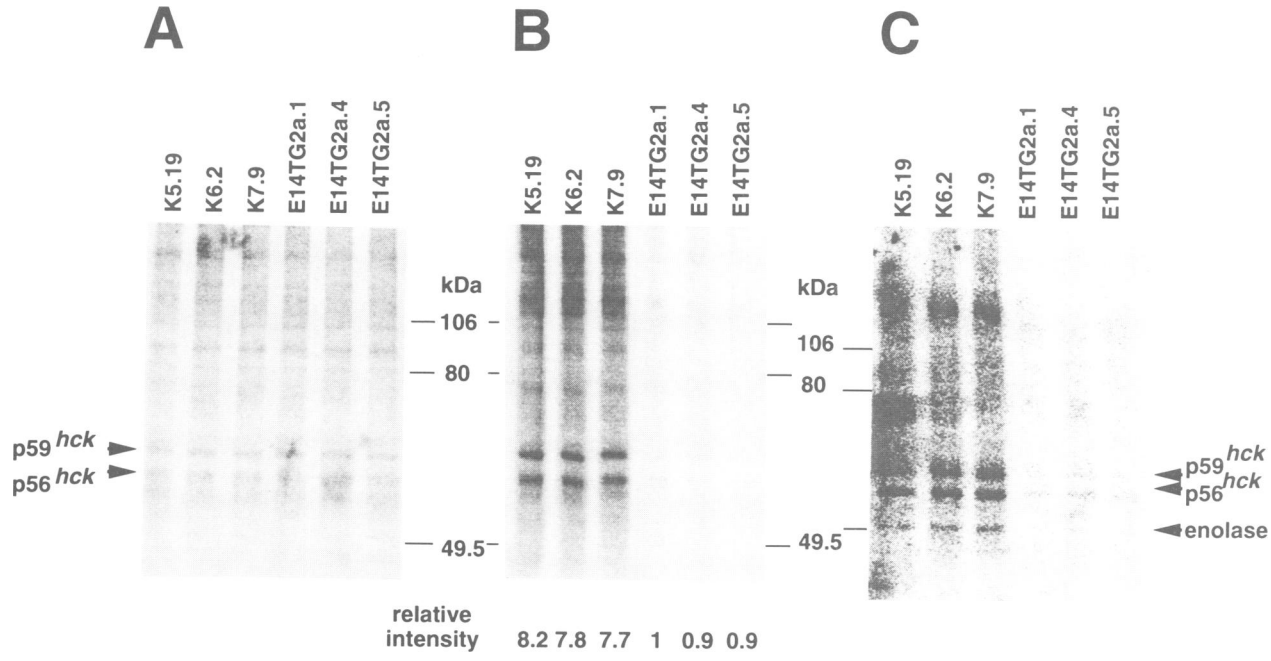

Fig. 3. Assessment of *in vitro* Hck kinase activity in targeted cell lines. Targeted (K5.19, K.6.2 and K7.9) or parental ES cells (E14TG2a.1, E14TG2a.4 and E14TG2a.5) were metabolically ^{35}S-labelled and 80% of each immunoprecipitated sample was subjected to SDS–PAGE (**A**). Of the remaining 20% of each sample, half was used for *in vitro* protein kinase assays to analyse for Hck autophosphorylation (**B**). The radiolabelled bands corresponding to the two Hck isoforms were quantified on a PhosphorImager. The relative intensity of the ^{32}P-labelled bands associated with p56/59^{hck} was calculated by normalizing the ^{32}P signal for the intensity of the corresponding ^{35}S signal, and this value, obtained from E14TG2a.1 cells, was then set as 1. The remainder (10%) of each sample was assayed for Hck specific kinase activity on exogenous substrates using enolase (**C**).

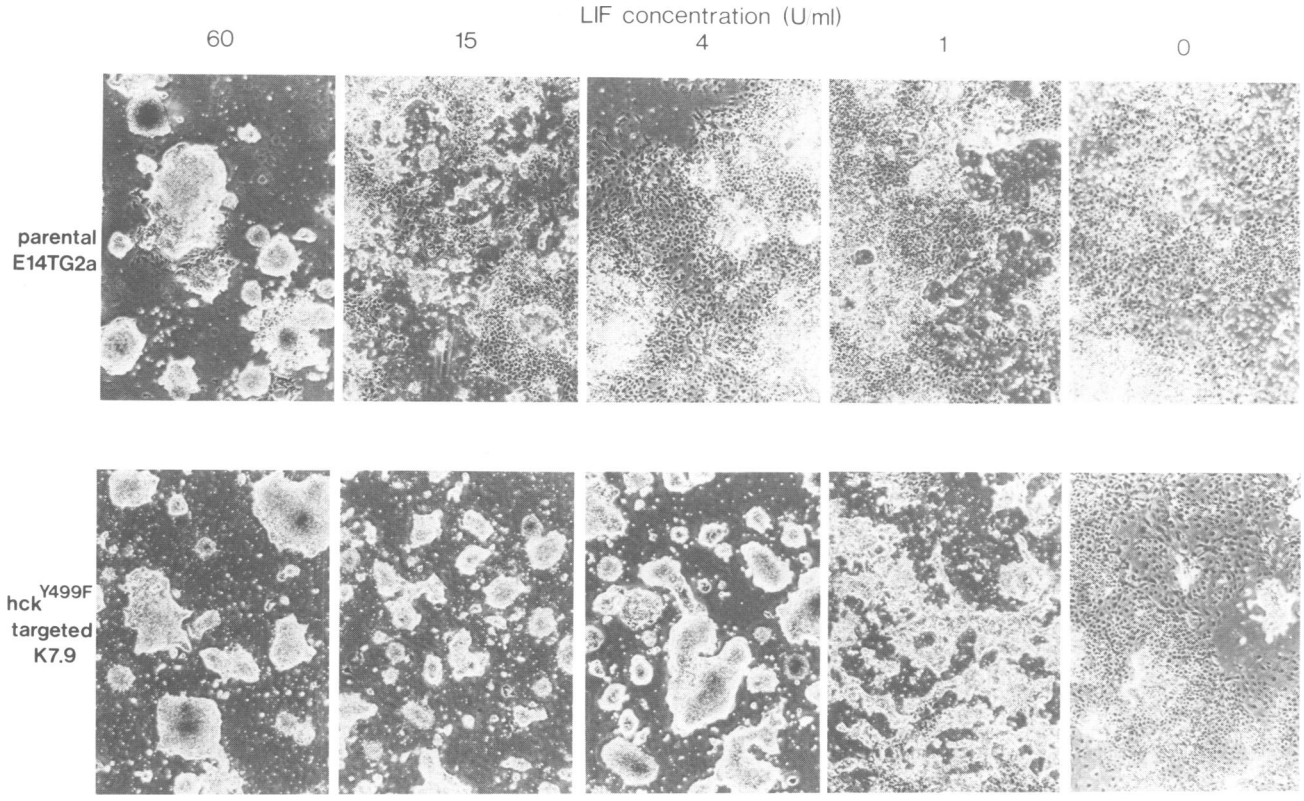

Fig. 4. Morphological appearance of targeted ES cells in response to decreasing concentrations of LIF. The parental cell line E14TG2a (**top row**) and the targeted HckY499F cell line K7.9 (**bottom row**) were plated in ES cell medium supplemented with 1000 U/ml LIF. Ten hours later, the amount of LIF was adjusted to the indicated concentration and the cultures were maintained therein for 96 h. The photomicrographs were taken at the end of the culture period.

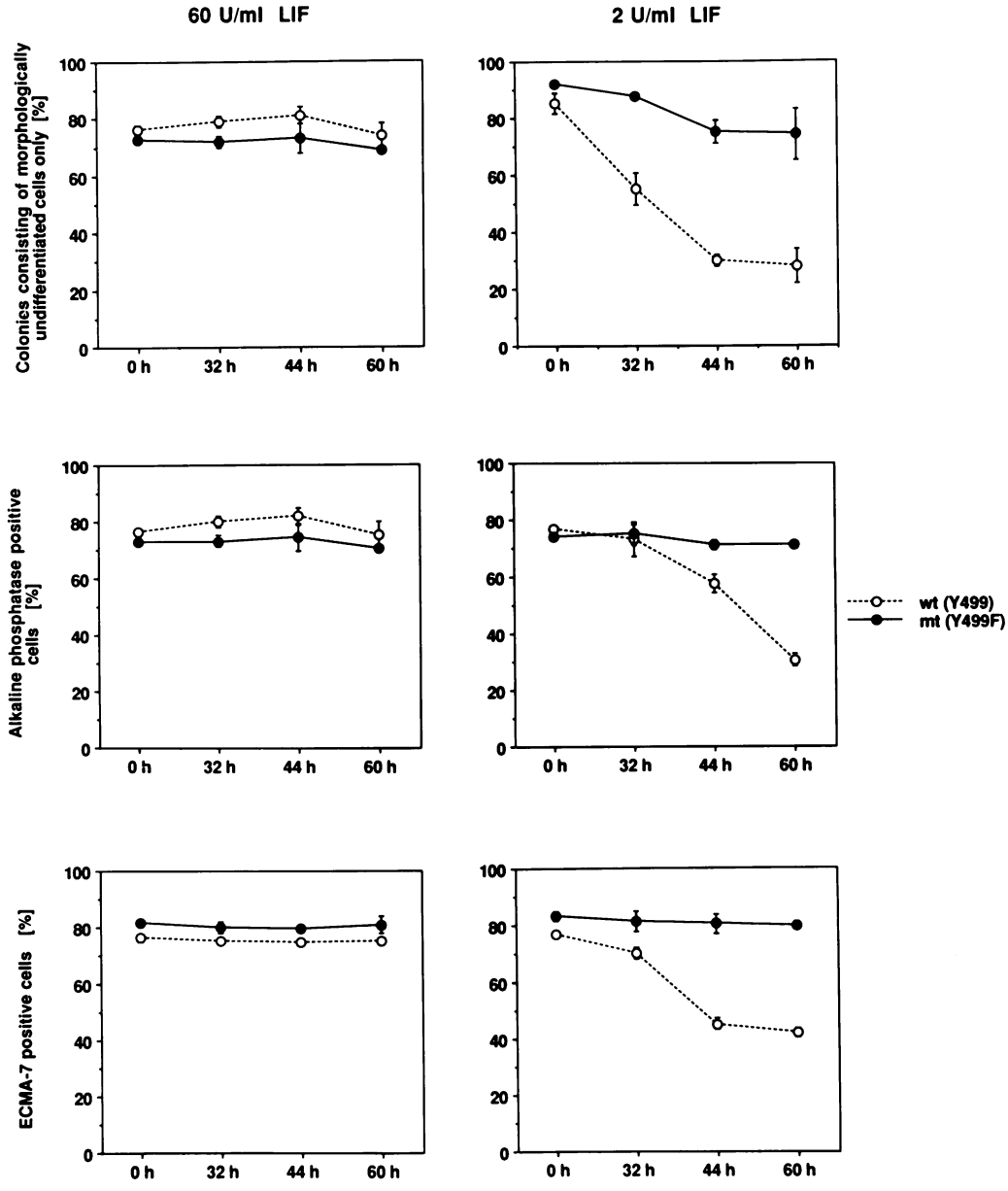

Fig. 5. Stem cell characteristics of ES cells following 'LIF-rescue'. Following a 32, 44 or 60 h 'LIF-starvation' in 60 U/ml LIF or 2 U/ml LIF and subsequent 'rescue', ES cell colonies were scored for morphology (**top**) as well as for alkaline phosphatase (**middle**) and ECMA-7 staining (**bottom**) of single cells from subsequently prepared cytospins. Staining for alkaline phosphatase was scored on the basis of the amount of cytoplasm occupied by the dye precipitate (Kaplow, 1955). Cells in which the cytoplasmic membrane stained brightly for ECMA-7 were scored positive whereas cells showing only dull staining of the cytoplasm were scored ECMA-7-negative. Each point represents the mean ± SD of values obtained for either three targeted (K5.19, K.6.2 and K7.9) or three parental (E14TG2a.1, E14TG2a.4 and E14TG2a.5) ES cell lines.

compared with ~75% in targeted cells. To confirm biochemically the stem cell character of the morphologically undifferentiated cells in the 'LIF-rescue' assay, the colonies were trypsinized at the end of the experiment and individual cells were analysed for expression of alkaline phosphatase and the stem cell specific surface antigen ECMA-7 (Boulter and Wagner, 1988). The fraction of alkaline phosphatase and ECMA-7-positive cells was comparable to the proportion of morphologically undifferentiated ES cells (Figure 5), indicating that a significantly higher proportion of ES cells expressing constitutively activated Hck tyrosine kinase remained undifferentiated than was observed with parental ES cells.

Hck is part of the LIF signal transduction pathway in ES cells

To evaluate further whether Hck is part of a LIF signal transduction pathway, we examined the enzymatic activity of Hck in ES cells stimulated with LIF. Cultures of ES cells were grown for 12 h in the absence of LIF (a period of time which is too short to induce irreversible cellular differentiation *in vitro* and to influence the level of *hck* transcription) and then transferred to culture medium supplemented with 1000 U/ml of LIF. Hck was immunoprecipitated from LIF stimulated ES cells and its tyrosine kinase activity assessed using an *in vitro* protein kinase assay. An increase in the tyrosine kinase activity of

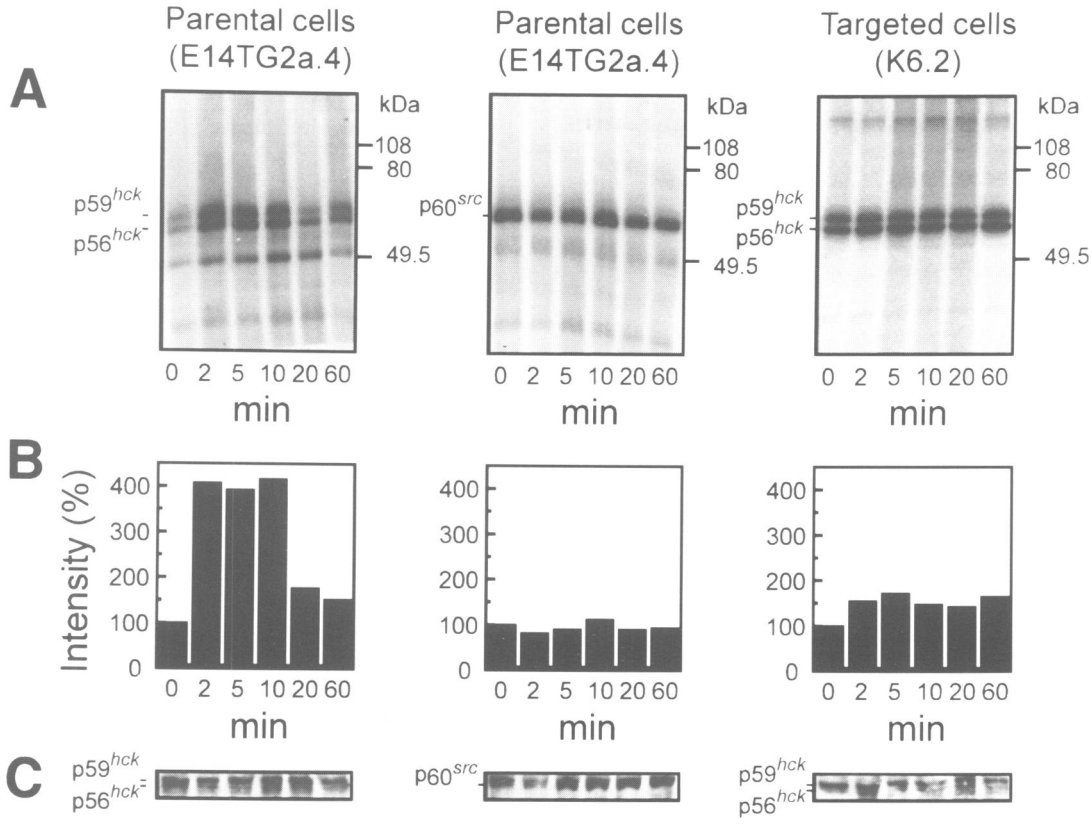

Fig. 6. Time course of LIF-dependent increase in Hck autophosphorylation activity. Cultures of undifferentiated parental (E14TG2a.4, left and middle panels) and targeted (K6.2, right panel) ES cells were stimulated with 1000 U/ml of LIF for the indicated amount of time. Membrane fractions of the cell lysates were then analysed for autophosphorylation activity following immunoprecipitation with either the anti-Hck antiserum (**A**, left and right panels) or the anti-Src antibody mAb327 (A, middle panel) and the intensity of radiolabelled p56/p59^{hck} or p60$^{c\text{-}src}$ was quantified on a PhosphorImager (**B**). The same volumes of cell lysate used for the immunoprecipitations were also used to determine the amount of Hck and Src protein by immunoblotting with either an immunoaffinity-purified anti-Hck antiserum (1077) or the mAb327 anti-Src antibodies and horseradish peroxidase coupled secondary antibodies (**C**).

p56/p59^{hck} in parental ES cells was observed within 2 min, reached maximal levels by 5−10 min and decreased thereafter (Figure 6A and B, left panels). Over the same time course, stimulation of ES cells with LIF did not alter substantially the relative amount of Hck protein as assessed by immunoblotting (Figure 6C, left panel). The LIF-dependent activation appears to be specific for Hck, since the tyrosine kinase activity of p60$^{c\text{-}src}$ (the only other Src kinase that is expressed in ES cells at levels similar to that of Hck) recovered from the same extracts, was unchanged (Figure 6A−C, middle panels). In contrast to parental ES cells, only a small elevation in Hck autophosphorylation was observed in targeted ES cells (Figure 6A−C, right panels) reflecting the fact that one *hck* allele includes the HckY499F mutation and hence encodes a constitutively activated kinase.

Association of Hck with the LIFR complex

For a number of cell surface non-tyrosine kinase receptors it has been possible to demonstrate by immunoprecipitation that one or several members of the Src family of protein tyrosine kinases is physically associated with the receptor complex and that the kinase is activated upon ligand binding. Since we were unable to determine whether Hck is physically associated with the murine LIFR (through the lack of a suitable antiserum directed against the murine LIFR) we generated an ES cell line (LIF-R5) expressing the human LIFR α-chain (see Materials and methods) which corresponds to the ligand binding subunit of the functional LIFR complex (Gearing *et al.*, 1991) and for which a monoclonal antiserum, M3, was available. Following immunoprecipitation with the M3 antiserum of ES cell lysates prepared from LIF-R5 cells, we investigated kinase activity of the immunoprecipitates by an *in vitro* protein kinase assay in the presence of [γ-^{32}P]ATP. As shown in Figure 7A, two major radiolabelled species were identified which comigrated with p56/59^{hck} immunoprecipitated from the same cell lysates using the Hck specific antiserum. By contrast, these radiolabelled bands were not observed when cell lysates from either undifferentiated parental ES cells or differentiated LIF-R5 cells (where Hck is transcriptionally down-regulated) were immunoprecipitated with the M3 antiserum and subjected to an *in vitro* kinase assay. To confirm the identity of the radiolabelled proteins immunoprecipitated from LIF-R5 cells using the M3 antiserum, the 56 and 59 kDa species were excised from the gel and subjected to limited digestion with staphylococcal V8 protease. The pattern of radiolabelled V8 fragments was identical to that generated by partial cleavage of p56/p59^{hck} immunoprecipitated from ES cells using the Hck specific antiserum (Figure 7B). Thus the 56 and 59 kDa species immunoprecipitated with the M3 anti-LIFR α-chain antiserum are p56/p59^{hck}.

The functional LIFR complex is known to be a

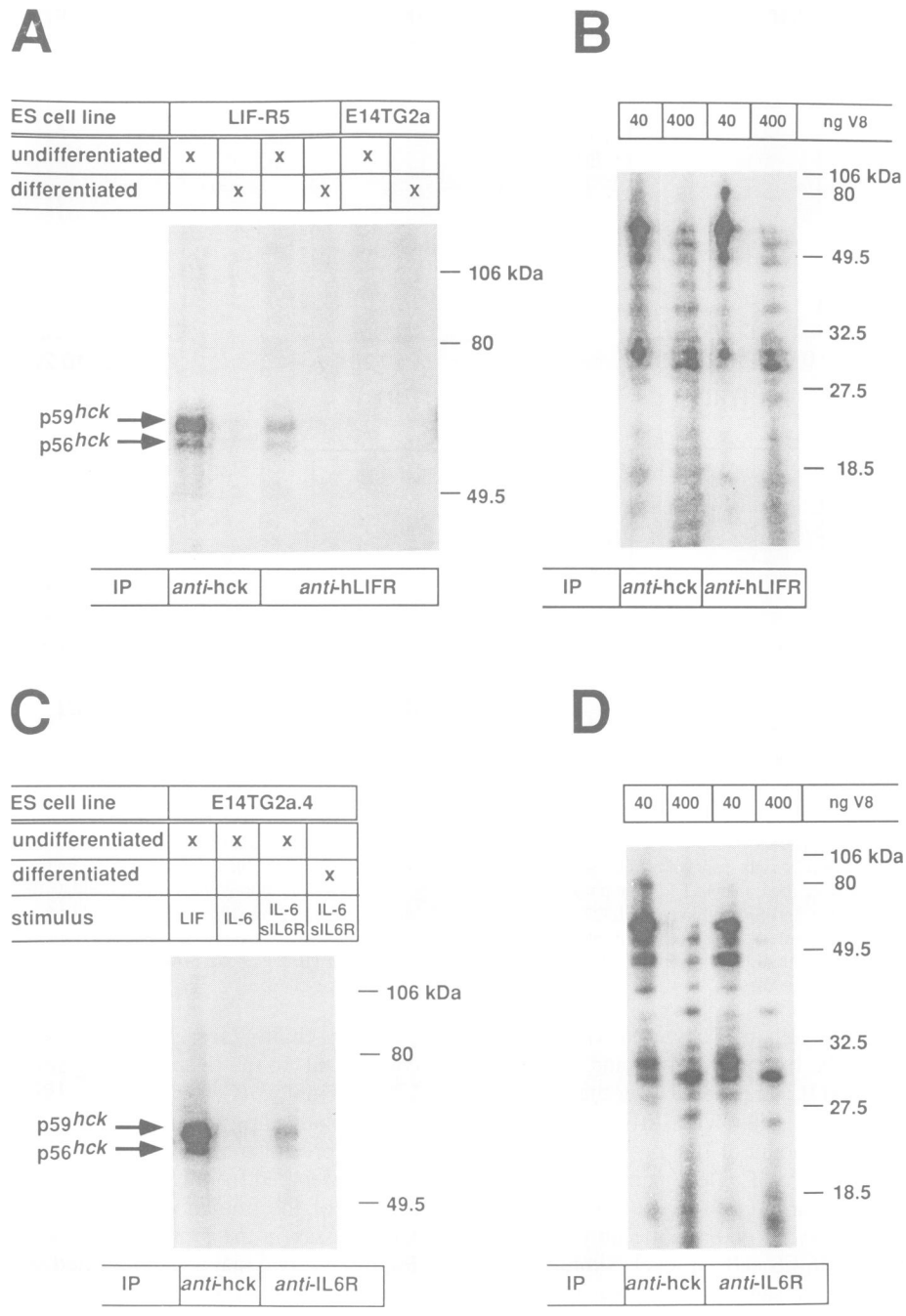

Fig. 7. Physical association of Hck with the gp130 signal transducer molecule in ES cells. Cultures of undifferentiated (maintained in LIF) or differentiated (grown for 96 h in the absence of LIF) parental ES cells (E14TG2a) or ES cells overexpressing the human LIFR α-chain (LIF-R5) were stimulated with either 1000 U/ml of LIF or 5 μg/ml of sIL-6R plus 500 ng/ml of IL-6 for 30 min. Cell lysates were immunoprecipitated with either a monoclonal antiserum directed against the human LIFR α-chain or an anti-Hck antiserum (**A** and **B**), or antisera raised against the IL-6R and Hck (**C** and **D**), followed by incubation with [γ-^{32}P]ATP and separation by SDS–PAGE (A and C). The phosphorylated bands in A and C with apparent mol. wts of 56 and 59 kDa were excised and subjected to limited proteolysis with the indicated amounts of staphylococcal V8 protease during SDS–PAGE (B and D). The radiolabelled bands were visualized using a PhosphorImager.

heterodimer of two transmembrane proteins consisting of the LIFR α-chain which displays low affinity binding to LIF (Gearing *et al.*, 1991), and the affinity converting/signal transducing subunit gp130 (Gearing *et al.*, 1992; Ip *et al.*, 1992). The latter protein serves as a common subunit in a number of cytokine receptor complexes including that for IL-6 (Hibi *et al.*, 1990). Although neither LIF nor IL-6 binds gp130 directly, the gp130 subunit is required for signal transduction via the LIFR and IL-6R complexes (Taga *et al.*, 1992). We thus sought to identify the LIFR complex subunit associating with Hck by immunoprecipitating a biologically active receptor complex in which gp130 is the only transmembrane molecule. Since undifferentiated ES cells lack binding sites for IL-6 (Saito *et al.*, 1992; M.Ernst, unpublished observations), ES cells were stimulated with IL-6 plus a non-membrane anchored soluble IL-6R (sIL-6R) (Taga *et al.*, 1992), a mixture which can maintain ES cells in an undifferentiated state in the absence of LIF (Saito *et al.*,

1992; M.Ernst, unpublished observations). Immunoprecipitation of cell lysates with a polyclonal antiserum raised against the extracellular domain of the IL-6R followed by an *in vitro* kinase assay again revealed two major species of radiolabelled proteins co-migrating with p56/p59^{hck} immunoprecipitated from the same cell lysates using the Hck specific antiserum (Figure 7C). The 56 and 59 kDa species were not present in cell lysates prepared from undifferentiated ES cells stimulated with IL-6 alone or from differentiated ES cells incubated with sIL-6R plus IL-6. The 56 and 59 kDa species immunoprecipitated using either the Hck or the IL-6R antiserum were excised from the gels and subjected to digestion with V8 protease. The pattern of V8-generated protein fragments obtained with proteins immunoprecipitated with either antiserum was identical (Figure 7D) and thus in ES cells, Hck is physically associated with gp130, the common signal transducing molecule in the LIFR and sIL-6R complexes.

Discussion

LIF is a multifunctional cytokine that acts on a wide range of cell types. In some cases the action of LIF serves to promote cellular differentiation, for example monocytic differentiation of M1 leukaemic cells (Hilton *et al.*, 1988), while in other cell types, such as ES cells, LIF promotes proliferation and suppresses differentiation (Smith *et al.*, 1988; Williams *et al.*, 1988). The action of LIF is mediated by low affinity binding to the LIFR α-chain which associates with the auxiliary transmembrane protein gp130, thereby generating high affinity binding sites for LIF (Gearing *et al.*, 1992). Although the LIF-dependent interaction between these receptor subunits and their subsequent tyrosine phosphorylation (Lord *et al.*, 1991; Davis *et al.*, 1993) is believed to be critical for biological signalling, the molecules that participate in LIF-mediated signal transduction have remained largely unknown.

Signal transduction from the LIFR is mediated by Hck

In the present study we have provided biological and biochemical evidence indicating that signal transduction via the LIFR complex is mediated, at least in part, through the Src-related kinase Hck. We have shown that targeted ES cells expressing elevated levels of Hck tyrosine kinase activity, retain their stem cell character when cultured in growth medium containing 15 times less LIF than that required to preserve the stem cell characteristics of parental ES cells. The dramatic reduction in the amount of ligand required for complete suppression of differentiation of HckY499F ES cells which express constitutively activated Hck is reminiscent of the increased sensitivity in T-cell receptor-dependent activation of thymocytes isolated from transgenic mice which overexpress an activated form of the Src-related tyrosine kinase, p56^{lyn} (Cooke *et al.*, 1991).

We have also demonstrated that stimulation of ES cells with LIF results in a rapid and transient increase in Hck tyrosine kinase activity. The kinetics of this induction are very similar to those observed for other Src family member kinases whose association with particular cell surface receptors is well established e.g. in the activation of p56^{lck} in T-cells following stimulation of the IL-2R by IL-2 (Hatakeyama *et al.*, 1991; Minami *et al.*, 1993) or following antibody-mediated cross-linking of CD4 (Veillette *et al.*, 1989), and of p53/p56^{lyn} following stimulation of myeloid leukaemic cells with IL-3 (Torigoe *et al.*, 1992). Furthermore, the difference in the magnitude of LIF-dependent Hck activation observed between parental and targeted ES cells strongly implies that phosphorylation of HckY499 is critical for the control of Hck tyrosine kinase activity *in vivo*. Our demonstration that, in the same cell lysates where the levels of p56/59^{hck} tyrosine kinase are elevated, the specific activity of p60$^{c\text{-}src}$ is unaltered following stimulation of ES cells with LIF confirms the specificity and likely relevance of the modulation of Hck tyrosine kinase activity in regulating ES cell differentiation.

Historically, the most compelling evidence for a functional link between a Src-related kinase and a cell surface receptor is inferred from the demonstration that a receptor and the Src kinase exist in the same physical complex (Veillette *et al.*, 1989; Samelson *et al.*, 1990; Burkhardt *et al.*, 1991; Hatakeyama *et al.*, 1991; Kobayashi *et al.*, 1993). Our demonstration that Hck can be immunoprecipitated from ES cells using the M3 antiserum raised against the LIFR α-chain is therefore consistent with this concept of 'guilt by association'. Interestingly, the immunoprecipitates obtained with the M3 LIFR α-chain antiserum contain only two major molecular species that are radiolabelled in the *in vitro* protein kinase assay, and we have shown these to be the two isoforms of Hck. While it seems likely that p56/p59^{hck} are radiolabelled by autophosphorylation we cannot rule out the possibility that the native LIFR complex includes other protein kinases that may contribute to the phosphorylation of Hck *in trans*. Indeed, the physical and functional association of more than one cytoplasmic tyrosine kinase to a particular transmembrane molecule has been observed for instance in the case of the IL-2R (Minami *et al.*, 1993) and the anti-immunoglobulin receptor in B-cells (Burkhardt *et al.*, 1991). If kinases other than Hck are contained within the LIFR complex immunoprecipitates, then they are neither autophosphorylated *in vitro* nor substrates for the Hck tyrosine kinase under our experimental conditions. Alternatively, additional kinases might form part of the LIFR complex *in vivo*, but their association is of sufficiently low affinity that they are lost during immunoprecipitation of the native LIFR complex despite the mild conditions of immunoprecipitation employed in the present study.

The notion that Hck is necessary but not sufficient for mimicking a full LIF signal is strongly suggested by our observation that ES cells expressing constitutive Hck tyrosine kinase are only partially released from their dependence on LIF for the suppression of differentiation. Intriguingly, ES cells expressing viral Src (a constitutively activated and somewhat promiscuous tyrosine kinase) fail to differentiate when grown without feeder cells or in growth medium lacking LIF (Boulter *et al.*, 1991). Possibly, the intracellular targets of the tyrosine kinase encoded by the viral *src* gene include all the physiological substrates required for a full LIF signal. These molecules may be part of potentially diverging signal transduction cascades that are activated separately by Hck and other tyrosine kinase(s) associated with the LIFR complex. The requirement for signalling through two diverging pathways in order to generate a full biological signal is reminiscent of the observation that full activation of IL-2 gene transcription requires both T-cell receptor-mediated activation of Ras and protein kinase C

(Rayter *et al.*, 1992; Woodrow *et al.*, 1993). Interestingly, the recently identified JAK-TYK family of cytoplasmic tyrosine kinases has been shown to be physically associated with some members of the haematopoietin superfamily of receptors (Argetsinger *et al.*, 1993; Silvennoinen *et al.*, 1993; Witthuhn *et al.*, 1993) which includes the LIFR α-chain as well as gp130. The JAK-TYK kinases are thought to transduce signals through entirely different pathways from Src-related tyrosine kinases, namely by the recruitment of latent cytoplasmic transcription factors of the ISGF-3α family by tyrosine phosphorylation and subsequent nuclear translocation (Hunter, 1993). It remains to be established whether the JAK-TYK kinases are functionally involved in the transduction of a LIF signal; however, we have observed a transient activation of JAK1 and JAK2 following LIF or IL-6 plus sIL-6R stimulation of ES cells *in vitro* (M.Ernst, unpublished observations).

Hck is associated with gp130

Like several other members of the haemopoietin family of cytokines (Liu *et al.*, 1992; Davis *et al.*, 1993; Stahl and Yancopoulos, 1993), both IL-6 (Hibi *et al.*, 1990) and LIF (Gearing *et al.*, 1992; Ip *et al.*, 1992) employ gp130 as a common high affinity and signal transducing β-chain. In the case of the IL-6R it is well established that gp130 does not bind IL-6 directly (Taga *et al.*, 1992), but it does bind the occupied IL-6R α-chain to form a high affinity IL-6R complex that initiates signal transduction (Hibi *et al.*, 1990). It has also been shown that a high affinity receptor complex can be generated when IL-6 and sIL-6R associate with gp130 (Taga *et al.*, 1989). Importantly while such a cell surface receptor complex is functional, only its gp130 subunit(s) extend through the cell membrane into the cytoplasm. Our demonstration therefore, that $p56/59^{hck}$ can be immunoprecipitated from ES cells treated simultaneously with IL-6 and sIL-6R shows unambiguously that, in the sIL-6R complex, gp130 is the molecular partner of Hck. Since exposure of ES cells to either LIF or IL-6/sIL-6R results in the suppression of differentiation and since in both of these receptor complexes gp130 serves as a common signal transducing β-chain, it seems probable that in the LIFR complex, Hck is the molecular partner of gp130. By the same token, it seems unlikely that Hck is also directly associated with the LIFR and IL-6R α-chains, although the design of our immunoprecipitation experiments does not categorically rule out this possibility. Whether gp130 and the LIFR α-chain serve as physiological substrates for Hck remains to be established since our immunoprecipitations from ES cells do not consistently show phosphorylated products corresponding to these receptor components. By contrast, their phosphorylation has been observed and been used to monitor ligand-dependent activation in COS cells transfected with the individual components of the LIF, IL-6 and ciliary neurotrophic factor (CNTF) receptor complexes (Davis *et al.*, 1993; Murakami *et al.*, 1993). It will be intriguing to establish whether the interaction between Hck and gp130 is ligand independent in a similar fashion to the association of JAK2 kinase molecules with some members of the haematopoietin receptor family (Argetsinger *et al.*, 1993; Witthuhn *et al.*, 1993).

We have recently observed that treatment of ES cells with oncostatin-M, the only known ligand for gp130 (Liu *et al.*, 1992), results in a similar transient increase in Hck tyrosine kinase to that observed in LIF-stimulated ES cells (M.Ernst, unpublished results) and prevents their differentiation. This observation raises the possibility that signalling through all receptor complexes that utilize gp130 as a common β-chain [e.g. LIF, IL-6 and IL-11 (Yin *et al.*, 1993), oncostatin-M and CNTF (Davis *et al.*, 1993)] is mediated in part by modulation of Hck tyrosine kinase. This may be the case in cells and tissues in which Hck and gp130 are co-expressed and may account, for instance, for the functional similarities of LIF and IL-6 (Hilton, 1992). However, Hck is unlikely to be the molecular partner of gp130 in all cells since, the expression of Hck is apparently restricted to ES cells and haematopoietic cells (Holtzman *et al.*, 1987; Quintrell *et al.*, 1987; Ziegler *et al.*, 1987), while gp130 is expressed in a wide variety of cell types and tissues (Saito *et al.*, 1992). Where Hck and gp130 are not co-expressed it is possible that another member of the Src family of protein tyrosine kinases is associated with gp130 and contributes to signal transduction from the receptor complex. Thus specificity in the signal transduction pathways employed by cytokines signalling through gp130 could still be achieved by the tissue specific expression of the ligand binding α-chain within the receptor complex and the signalling molecules associated with an activated receptor complex.

Materials and methods

Targeting vector

A mouse BALB/c-derived genomic fragment encompassing 2.2 kb of intronic sequence 5′ of the 3′-terminal coding *hck* exon and 6.6 kb of 3′-flanking sequence (Holtzman *et al.*, 1987; Ziegler *et al.*, 1991) was cloned into the *Bam*HI and *Xho*I sites of the pIC-20H vector. To allow easy screening for homologous recombination by the polymerase chain reaction (PCR), the most 5′ *Bam*HI−*Spe*I fragment was deleted and the *Spe*I site was fused to the *Xba*I site within the polylinker. Site directed mutagenesis using a PCR-based strategy was performed yielding a 532 bp fragment containing the mutated sequence 5′-**GCG**,**ACA**,**GAG**,**TCG**,**CAA**,*T*T*T*-3′ (conservative changes in bold, Y499F mutation in italics) which was then replaced with a 191 bp fragment, defined by a *Sac*I site (5′-end) and *Apa*I site (3′-end) adjacent to the mutation (Figure 1). A 1.9 kb *Nae*I−*Eco*RI fragment of the murine HPRT minigene consisting of exons 1, 4, 7, 8 and 9 (Melton *et al.*, 1986) was ligated into the murine PGK expression cassette (gift of Dr A.Berns), and was subsequently cloned as a 3.0 kb fragment into the unique *Sal*I site in the pIC-20H polylinker at the 5′-end of the *hck* sequence, yielding the targeting plasmid p*HCK*499F.

Generation of targeted HckY499F cell lines

Fifteen micrograms of plasmid p*HCK*499F were linearized with *Eco*RI at the unique restriction site within the region of homology and electroporated (500 μF, 270 V; Bio-Rad Genepulser) into 2.5×10^7 E14TG2a ES cells. This previously isolated ES cell line (Hooper *et al.*, 1987) was established as a spontaneous mutation in culture and carries a 10 kb deletion encompassing the promoter and first two exons (Thompson *et al.*, 1989) of the X-linked HPRT gene, rendering the cells phenotypically $hprt^-$. Electroporated E14TG2a cells were plated into ten 250 ml culture flasks (Nunc) onto irradiated (3000 rad, 20 min) STO feeder cells in ES cell medium (Dulbecco's modified Eagle's medium containing 15% fetal calf serum and 0.1 mM 2-mercaptoethanol) plus 1000 U/ml LIF (Esgro from Gibco). Twenty-four hours after electroporation, the cultures were selected in HAT-containing ES cell medium (120 μM hypoxanthine, 0.4 μM aminopterine, 20 μM thymidine) for 8 days. As the reversion rate of a duplication of homology is proportional to the total number of targeted and thus HAT^r cells, the total number of HAT^r cells was amplified while being grown for one passage on STO feeder cells without further selection. This allowed spontaneous revertants time to purge residual HPRT mRNA or protein (Valancius and Smithies, 1991). HAT^r cells, plated at 2.5×10^3 cells/cm^2, were subsequently selected for revertants in the absence of STO feeder cells in ES cell medium containing 10 μM 6-thioguanine (6-TG) for an additional 8 days.

Identification and genetic analysis of targeted Hck cells

DNA was prepared from pools of 6-TG^r colonies by incubating the cultures for 3 h at 37°C in lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) containing 150 μg/ml Pronase (Boehringer Mannheim). DNA was precipitated with isopropanol and resuspended in distilled water to a concentration of ~50 ng/μl. Five microlitres of genomic DNA were used for PCR analysis to screen for the presence of the mutations employing a 24mer sense primer outside of the 5′-end of the targeting construct (primer 1, 5′-AGG,AAA,TGA,TGG,TGT,TCA,GAG,TGA-3′; Figure 2) and a 24mer antisense primer directed against the mutation (primer 2, 5′-TCA,AGG,CTG,CTG,CTG,AAA,**TTG,CGA**-3′, mutated sequence in bold; Figure 1). The PCRs were performed using Taq polymerase (Biotech, Australia) in the presence of 2.5 mM $MgCl_2$ in a thermal cycler for an initial incubation at 95°C (100 s), followed by 35 cycles of 95°C (50 s), 60°C (50 s) and 72°C (50 s). Individual 6-TG^r colonies were then picked from 6-TG^r-positive pools and expanded in 24-well plates, and DNA of individually isolated 6-TG^r colonies was analysed by PCR for the presence of the mutations.

For nucleotide sequencing of the targeted *hck* exon and of the flanking upstream intron, PCR-positive colonies were amplified for two passages without STO cells in gelatin-coated culture dishes. DNA was then prepared and PCRs were performed with primer 1 and primer 3 (5′-ATA,CCA,ACT,TGG,AGG-3′) which maps 302 bp downstream of the Y499F mutation. The PCR products were subcloned and the recombinant plasmids were sequenced on an automated fluorescent sequencing system (Applied Biosystems).

Metabolic labelling, in vitro kinase assay and immunoblotting

Targeted and parental ES cell lines, previously grown for two passages in the absence of STO feeder cells, were plated at 5×10^3 cells/cm^2 in gelatin-coated 150 mm diameter culture dishes (Nunc). Cultures of undifferentiated cells (~2×10^7) were then incubated for 12 h in methionine/cysteine-free Dulbecco's modified Eagle's medium (Flow Laboratories) containing 15% dialysed fetal calf serum and 1 mCi of [^{35}S]methionine/[^{35}S]cysteine (Expre^{35}S^{35}S labelling mix from New England Nuclear) corresponding to 5% (v/v) of the incubation medium. The cells were harvested in ice-cold phosphate-buffered saline (PBS) and lysed in 500 μl modified LAU buffer [0.5% sodium deoxycholate, 1% NP-40, 100 mM NaCl, 10 mM Tris pH 7.5, 1 mM phenylmethylsulfonyl-fluoride (PMSF), 1% Trasylol, 0.1 mM Na_3VO_4] for 30 min on ice before removing the nuclei by centrifugation. The protein concentration of the supernatant was determined using a BCA kit (Pierce), and aliquots of 150 μg protein were pre-cleared twice with Pansorbin (Calbiochem) and immunoprecipitated with the Hck preimmune serum. The samples, containing approximately identical amounts of incorporated ^{35}S radioactivity, were then immunoprecipitated for 3 h at 4°C in the presence of 1% bovine serum albumin (BSA) with an Hck-specific antiserum [1:200 dilution (Boulet *et al.*, 1992)] and protein A−Sepharose as a carrier, and the immunoprecipitates were washed twice in modified LAU buffer. Eighty percent of each sample was loaded directly onto an 10% SDS−polyacrylamide gel and half of the remainder of each sample was used in a protein kinase assay by adding 100 μl of kinase buffer [20 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 0.5% NP-40, 0.1 mM Na_3VO_4] containing 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Bresatec, Australia) and incubating the reaction for 10 min at 25°C. All samples were separated on a reducing 10% SDS−polyacrylamide gel. The gel was dried without further treatment and exposed to a PhosphorImager screen, and the intensity of the signals associated with the bands was quantified using a PhosphorImager (Molecular Dynamics). In order to determine the kinase activity on the endogenous substrate enolase, aliquots of the metabolically ^{35}S-labelled cell lysates (in modified LAU buffer) were washed once in enolase buffer (100 mM HEPES pH 7.0, 10 mM $MnCl_2$, 1 mM PMSF, 1% Trasylol, 0.1 mM Na_3VO_4). Enolase (17 μg, Sigma) was resuspended in 20 μl buffer (50 mM HEPES pH 7.0, 1 mM DTT, 10 mM $MgCl_2$) and denatured at 30°C for 5 min by adding an equal volume of 25 mM acetic acid (pH 3.3). Following neutralization with 1 ml of enolase buffer, 60 μl of enolase solution containing 100 nM cold ATP and 10 μCi [γ-^{32}P]ATP was added to each sample and incubated at 30°C for 10 min. The denatured samples were fractionated on a 10% SDS−polyacrylamide gel which was subsequently treated with 1 M KOH for 2 h at 55°C to reduce binding of ^{32}P to serine and threonine residues.

For experiments investigating LIF-dependent Hck activation, cultures of undifferentiated ES cells were incubated for 12 h in ES cell medium lacking LIF before transferring back to ES cell medium supplemented with LIF (1000 U/ml). The cells were lysed in hypotonic buffer (2.5 mM Tris pH 7.5, 2.5 mM KCl, 0.5 mM $MgCl_2$, 1 mM PMSF, 1 mM ε-amino caproic acid, 1 mM DTT) and the membrane-containing fraction prepared by ultracentrifugation (Lock *et al.*, 1991). The membrane-containing pellets were resuspended in modified LAU buffer and 150 μg of protein was immunoprecipitated either with the anti-Hck antiserum (1:200 dilution) or with the monoclonal anti-Src antibody mAb327 (1:200 dilution, gift from Dr J.Brugge) and assayed for autokinase activity as described above. The denatured samples were separated on a 10% SDS−polyacrylamide gel which was then treated with alkali before being exposed to a PhosphorImager screen. For immunoblotting, 150 μg of membrane-containing samples were separated on a 10% SDS−polyacrylamide gel and electroblotted onto an Immobilon-P membrane (Millipore). The membrane was preincubated in blocking buffer (PBS containing 5% sheep serum, 0.5% BSA, 0.2% Tween-20) before adding the rabbit anti-mouse Hck antiserum 1077 (1:500 dilution, kind gift from Dr C.Lowell) or the mAb327 anti-Src antibody (1:1000 dilution). The bands were visualized following incubation with horseradish peroxidase coupled goat anti-rabbit IgG (Bio-Rad) and ECL reagents (Amersham).

The physical association of Hck with the LIFR complex was investigated in LIF-R5 ES cells, an E14TG2a-derived cell line stably transfected with a human LIFR α-chain cDNA cloned into the hygromycin selectable retroviral vector tgls(+)HyTK, or in cultures of parental E14TG2a ES cells which were incubated for 30 min with 5 μg/ml of human sIL-6R (encompassing amino acids 1−344 of the extracellular domain) (Yasukawa *et al.*, 1990) plus 500 ng/ml IL-6 (Zhang *et al.*, 1992). After rinsing, 2×10^7 cells were lysed on ice for 15 min in digitonin lysis buffer (1% digitonin, 150 mM NaCl, 10 mM triethanolamine pH 7.8, 10 mM iodoacetamide, 1 mM EDTA, 1 mM PMSF, 0.1 mM Na_3VO_4). The cell extracts were centrifuged at 10 000 *g* for 30 min and the supernatant was incubated for 16 h at 4°C with either the monoclonal antiserum M3 directed against the human LIFR α-chain (1:200 dilution) or with an IL-6R specific antiserum (1:200 dilution, kind gift of Dr K.Yasukawa) and protein A−Sepharose as a carrier. The immunoprecipitated pellets were washed four times in digitonin lysis buffer and then 100 μl of kinase buffer containing 10 μCi [γ-^{32}P]ATP was added and the reaction was incubated at 25°C for 30 min. Following separation of the denatured samples on a 10% SDS−polyacrylamide gel, the radiolabelled p56/p59^{hck} bands were excised from the dried gel and subjected to partial digestion with staphylococcal V8 protease (Sigma) during the course of electrophoretic separation through a 15% SDS−polyacrylamide gel (Cleveland, 1983).

Cell culture assays

Assays were performed with three independently derived targeted HckY499F ES cell lines (K5.19, K6.2 and K7.9) and three subclones of the parental cell line E14TG2a (E14TG2a.1 and E14TG2a.4 and E14TG2a.5). Prior to the 'LIF-rescue' assays, all cell lines were maintained for five passages independently of feeder cells. For 'LIF-rescue' assays, cells were plated at a density of 500 cells/cm^2 in gelatin-coated 6-well plates (Nunc) and maintained for 24 h in ES cell medium supplemented with 1000 U/ml of LIF. Subsequently, cultures were washed twice with ES cell medium and incubated for 20, 32, 44 or 60 h in ES cell medium supplemented with the indicated concentration of LIF. The LIF concentration was then raised back to 1000 U/ml. The proportion of undifferentiated colonies was calculated by inspecting the morphology of 300 randomly chosen colonies, 120 h after initial plating of the cells. Only colonies consisting entirely of densely packed cells were scored as undifferentiated, whereas colonies consisting of densely packed cells, as well as cells of more flattened morphology, were scored as differentiated colonies. At the end of the 'LIF-rescue' experiments, some cultures were trypsinized and cytospins were prepared which were stained for alkaline phosphatase activity. The proportion of alkaline phosphatase-positive cells was scored from a total of 300 cells in randomly chosen microscopic fields. ECMA-7 expression was monitored by analysing cytospins of ES cells which had been incubated with ascites fluid from a hybridoma cell line producing the monoclonal antibody ECMA-7 for 30 min on ice (Boulter and Wagner, 1988). After washing with PBS containing 1% BSA, cell preparations were incubated for 30 min with FITC-conjugated Fab′ fragments of a goat anti-mouse IgG (1:50 dilution, Amersham). The slides were then washed with PBS and the fraction of ECMA-7-positive cells was determined after the inspection of 300 cells in randomly chosen fields.

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